9-1991

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Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7815–7819, September 1991
Cell Biology

Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat

(cardiac muscle/diabetes/glucose transport/protein targeting/immunocytochemistry)

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Communicated by Harvey F. Lodish, May 30, 1991

ABSTRACT **The insulin-regulated glucose transporter GLUT4 was immunolocalized in rat cardiac muscle under conditions of basal and stimulated glucose uptake, achieved by fasting and a combined exercise/insulin stimulus, respectively. In basal myocytes there was very little (<1%) GLUT4 in the different domains of the plasma membrane (sarcolemma, intercalated disk, and transverse tubular system). GLUT4 was localized in small tubulo-vesicular elements that occur predominantly near the sarcolemma and the transverse tubular system and in the trans-Golgi region. Upon stimulation ≈42% of GLUT4 was found in the plasma membrane. Each domain of the plasma membrane contributed equally to this effect. GLUT4-positive, clathrin-coated pits were also present at each cell surface domain. The remainder of the labeling was in tubulo-vesicular elements at the same sites as in basal cells and in the intercalated disk areas. The localization of GLUT4 in cardiac myocytes is essentially the same as in brown adipocytes, skeletal muscle, and white adipocytes. We conclude that increased glucose transport in muscle and fat is accounted for by translocation of GLUT4 from the intracellular tubulo-vesicular elements to the plasma membrane. The labeling of coated pits indicates that in stimulated myocytes, as in adipocytes, GLUT4 recycles constantly between the endosomal compartment and the plasma membrane and that stimulation of the exocytotic rate constant is likely the major mechanism for GLUT4 translocation.**

Glucose transport in muscle and fat is acutely regulated by factors such as insulin and exercise (1, 2). These tissues express a unique insulin-regulatable glucose transporter, GLUT4, that is not found in other tissues. In the postabsorptive state, muscle is the primary site for insulin-stimulated glucose disposal (3). Thus the regulation of muscle glucose transport by insulin is a critical determinant of whole body glucose homeostasis. Most research concerning insulin regulation of glucose transport has focused on adipocytes. By using various techniques it has been shown that insulin stimulates glucose transport by means of translocation of GLUT4 from an internal pool to the plasma membrane (4–6). Due to a number of limitations it has been difficult to perform similar analyses in muscle. First, it is difficult to perform subcellular fractionation in muscle due in part to the presence of multiple surface membranes (7). These include the sarcolemma, the lateral membrane of the cell; the transverse tubular system (T system), the membrane that protrudes transversely (and axially in heart) through the muscle cells; and (only in heart) the intercalated disk (ID), the membrane adjacent to the sarcolemma that crosses the fibrillar texture of the tissue and that is occupied by many junctional specialties. Second, there is a lack of suitable muscle cell culture lines that exhibit insulin-stimulated glucose transport. Third, metabolic control is closely linked to innervation and/or contractility in muscle. Thus, glucose transport may be activated during muscle dissection. In the present study we have used immunocytochemistry, which overcomes many of these problems, to study the distribution of GLUT4 in basal and stimulated cardiac muscle. The data indicate a regulation of glucose transport similar to that previously observed in adipocytes.

MATERIALS AND METHODS

Male Wistar rats (150 g) were anesthetized (Nembutal, 90 mg/kg of body weight), and whole body fixation was performed by perfusion with 2% paraformaldehyde/0.2% glutaraldehyde. *Basal* animals were fasted overnight. *Stimulated* animals were not fasted and were injected intraperitoneally with a mixture of insulin (8 units/kg) and D-glucose (1 g/kg) 30 min prior to fixation and forced to exercise on a treadmill (24 m/min) during the last 25 min before fixation. Ultrathin cryosections were prepared from fixed cardiac tissue taken from the musculus papillaris and the left ventricle wall; these gave the same qualitative result with respect to GLUT4 distribution. Musculus papillaris was selected for quantitation (Table 1) because reproducible sampling was easier with it. For GLUT4 labeling the sections were incubated with antibodies against the C-terminal 19 amino acid residues of GLUT4, with swine anti-rabbit IgG serum (Nordic, Tilburg, The Netherlands) and then with protein A-gold. Subsequently, after treatment with 1% glutaraldehyde, most sections were labeled with rabbit anti-rat albumin followed again by protein A-gold and sometimes, again after glutaraldehyde treatment, labeled using rabbit anti-clathrin (kindly provided by E. Ungewickel, Munich) and protein A-gold. In double- and triple-labeled sections gold markers with sizes of 15, 10, and 5 nm were used for GLUT4, clathrin, and albumin labeling, respectively. Albumin labeling was used to mark endocytotic structures and the extracellular space, which was helpful in determining the cell border, particularly at the undulating ID surface (Fig. 1*D*), and to recognize the T system (Fig. 2 *A–D*). For quantitations (see Table 1) the swine anti-rabbit IgG step in the GLUT4 labeling procedure was omitted. Further details of fixation, immunoreagents, immunolabeling, and treatment of cryosections are described elsewhere (6).

Control sections were labeled with an irrelevant rabbit IgG instead of anti-GLUT4 or with anti-GLUT4 that was mixed 30 min prior to use with an excess of the peptide against which the antibody was raised. No specific labeling was observed in these sections.

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Abbreviations: GLUT4, isotype 4 of the glucose transporter; ID, intercalated disk; SR, sarcoplasmic reticulum; T system, transverse tubular system; TGR, trans-Golgi reticulum; T-V, tubulo-vesicular.

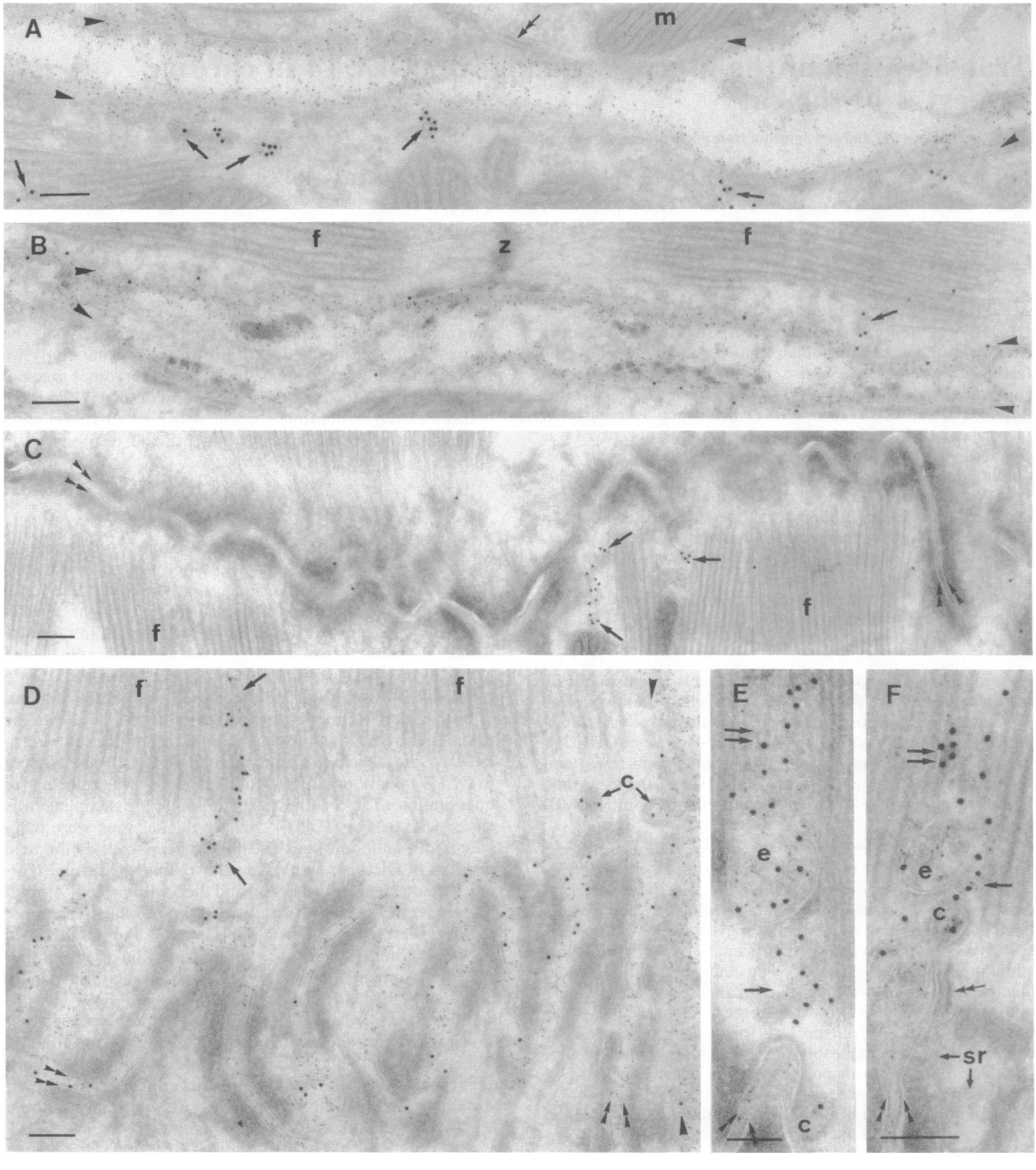

FIG. 1. Cryosections of basal (*A* and *C*) and stimulated (*B* and *D–F*) cardiac muscle, labeled for GLUT4 with 15-nm gold and, except *C*, for albumin with 5-nm gold. The panels demonstrate the increase of GLUT4 labeling in the lateral (*A* and *B*) and ID (*C* and *D*) domains of the plasma membrane from basal state (*A* and *C*) to stimulated (*B* and *D*) state of the myocytes. GLUT4-positive T-V elements occur more frequently near the lateral cell membrane in basal (*A*) than in stimulated (*B*) cells. In the ID areas, on the other hand, T-V elements are more common in stimulated cells (*D*) and here they often occurred in characteristic arrays that pierced in between the myofilaments. These GLUT4-positive arrays included small (200–500 nm) multivescular bodies identified as endosomal vacuoles (e) by their albumin labeling (*E* and *F*). Note that albumin labeling is also present in T-V elements at the plasma membrane side of the endosomes (single arrows), but to a less extent in similar structures at the opposite pole of the endosome (double arrows). GLUT4-representing gold particles were often associated with coated pits of lateral (*D*) and ID (*D–F*) plasma membrane. t, T system; c, coated pits; g, Golgi cisternae; f, myofilaments; z, Z discs; sr, SR; m, mitochondria; e, endosomes; ➤, sarcolemma; ➤➤, ID plasma membrane; →, T-V elements; →➤, junctional SR. (Bars = 200 nm.)

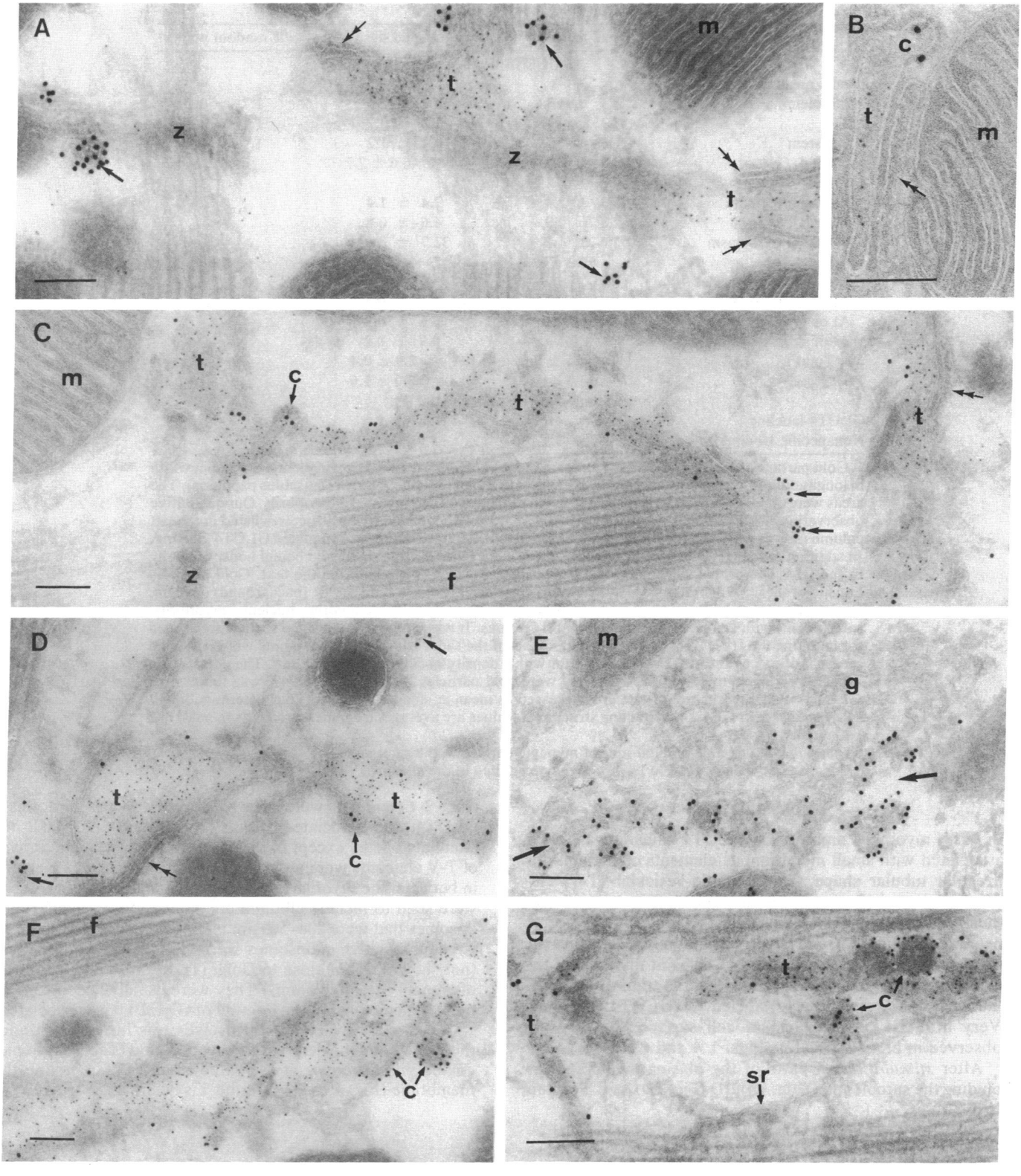

FIG. 2. Basal (*A*, *B*, and *E*) and stimulated (*C*, *D*, *F*, and *G*) cardiac cells, labeled for GLUT4 (15-nm gold), albumin (5-nm gold), and (only *F* and *G*) clathrin (10-nm gold). The albumin-positive tubules of the T system have very low GLUT4 labeling in basal cells (*A* and *B*), but occasionally labeled coated pits were attached to them (*B*). Many GLUT4-labeled T-V elements are seen in the cytoplasm, often near the T system elements (*A*). In stimulated cells, many GLUT4 markers were associated with the membranes of the T system membranes, labeled coated pits were often found, and some T-V elements still occurred in the cytoplasm (*C* and *D*). In *F* and *G*, two clathrin-labeled coated pits are indicated at the sarcolemma and T system of a stimulated cell, respectively. In each case, one is labeled for GLUT4. GLUT4-labeled elements in the TGR (⟶) of a basal cell are evident in *E*. No significant GLUT4 labeling was associated with the SR, either in basal (see Fig. 3) or in stimulated (*G*) cells. In addition, GLUT4-negative junctional elements of the SR are shown in basal (*A* and *B*) and stimulated (*C* and *D*) cells (see also Fig. 1 *A* and *F*). See legend to Fig. 1 for symbols. (Bars = 200 nm.)

Table 1. Distribution of GLUT4 in rat cardiac myocytes

GLUT4 distribution	Basal tissue	Stimulated tissue	% random points*
	% GLUT4 labeling		
Plasma membrane			
Sarcolemma	2.3 ± 0.2	18.7 ± 2.6	1.7 ± 0.1
ID	0.9 ± 0.3	7.7 ± 0.7	1.1 ± 0.2
T system	1.8 ± 0.5	20.4 ± 1.2	1.9 ± 0.1
Total	5.0 ± 0.4	46.6 ± 2.7	4.7 ± 0.2
T-V elements			
Near sarcolemma	26.3 ± 2.4	7.4 ± 1.4	—
Near ID	2.3 ± 0.9	4.6 ± 0.8	—
In rest of cytoplasm	52.6 ± 1.2	32.7 ± 3.6	—
Total	81.2 ± 2.1	44.5 ± 2.1	0.8 ± 0.1
Coated pits and vesicles			
At or near sarcolemma	0.4 ± 0.3	1.5 ± 0.4	—
At or near ID	0.1 ± 0.1	0.6 ± 0.3	—
At or near T system	<0.1	0.9 ± 0.2	—
Total	0.5 ± 0.3	2.9 ± 0.4	<0.1
TGR, total	13.3 ± 2.2	6.0 ± 1.6	0.2 ± 0.1
	Labeling density, gold per 100 μm^2		
GLUT4 labeling†	35.4 ± 4.1	34.6 ± 3.5	
Nonspecific labeling‡	38.5 ± 3.9	35.9 ± 5.0	

Gold particles over myocytes were counted in areas of about 1000 μm in length and 2.5 μm in width in longitudinally cut sections of four basal and four stimulated tissue samples (musculus papillaris). The areas were chosen at random, in longitudinal direction at a 45° angle with the myofibrils. Our qualitative observations demonstrate that the presence of GLUT4 was confined to the structures listed in the left column (see *Results*). Gold within 20 nm distance of these structures was designated GLUT4 labeling. In addition to the GLUT4-specific labeling, these values also include some gold bound nonspecifically to the GLUT4-positive structures, the significance of which is discussed in the text. Gold particles outside of these GLUT4-containing structures were considered as nonspecific (background) labeling. Background occurred evenly over other cellular structures (mitochondria, nucleus, myofibrils, sarcoplasm) with similar density as in control sections. It was counted in the same section areas used for counting the GLUT4 labeling. In similar areas of the same sections random points were projected on the sections by means of a video monitor with a density of 50 points per 100 μm^2. The points were assigned to myocyte structures as if they were gold particles. Approximately 94% was found over GLUT4-negative structures. Data are expressed as mean ± standard error for four determinations.

*Percentage over GLUT4-containing structures. Values are averages from measurements in basal and stimulated tissue.

†Total GLUT4 labeling per 100 μm^2 of myocyte surface in the sections.

‡Nonspecific labeling per 100 μm^2 of myocyte surface in the sections.

RESULTS

In *basal* myocytes most of the GLUT4 labeling was found associated with small membranous elements of vesicular or irregular tubular shape. These tubulo-vesicular (T-V) elements occurred often near the sarcolemma (Fig. 1*A*) and near the T system in the Z-line area (Fig. 2*A*), occurred less often near the ID (Fig. 1*C*), and occurred randomly throughout the sarcoplasm. Similar tubules and vesicles at one side of the Golgi cisternae, probably representing the trans-Golgi reticulum (TGR), were strongly labeled for GLUT4 (Fig. 2*E*). Very little GLUT4 labeling of cell surface domains was observed in basal myocytes (Figs. 1 *A* and *C* and 2*A*).

After *stimulation* all parts of the plasma membrane, including the sarcolemma (Fig. 1*B*), ID (Fig. 1*D*), and T system (Fig. 2 *C* and *D*), were clearly labeled for GLUT4. Less GLUT4 was associated with T-V elements and TGR in stimulated cells, except in the ID area, where typical arrays of T-V elements were more prominent. These arrays pierced in between the myofilaments (Fig. 1*D*), and sometimes they were seen to include albumin-containing multivesicular endosomes that were 200–500 nm in diameter (Fig. 1 *E* and *F*). Albumin-positive endosomes were also found at other sites (not shown), often close to Golgi (TGR) structures and less often near the sarcolemma. They were usually accompanied by T-V elements, but the fraction of GLUT4 label associated with endosomal vacuoles themselves was insignificant and therefore not considered in the quantitation (Table 1). Some coated pits occurring at the sarcolemma (Fig. 1*D*), the ID membrane (Fig. 1 *D–F*), and the T system (Fig. 2 *C* and *D*)

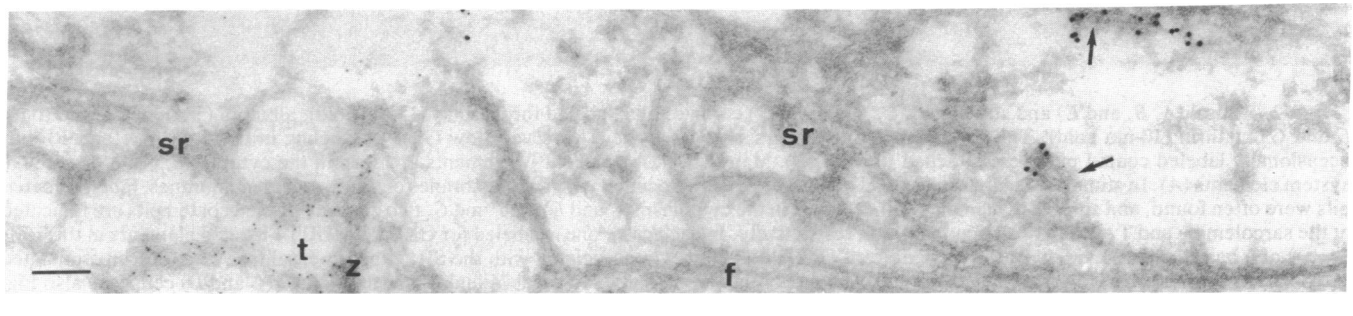

FIG. 3. Basal cardiac myocyte, labeled as in Fig. 1. GLUT4 (15-nm gold) is present in T-V elements (arrows) but not in the T system (t) and the SR network (sr). (Bar = 200 nm.)

were positive for GLUT4. In triple-labeled sections, in addition to the GLUT4 and albumin labeling, a clathrin decoration with 10-nm gold marked all such pits at the different plasma membrane domains (Fig. 2 *F* and *G*). GLUT4 was much less abundant in the coated pits of basal cells, but occasionally we encountered a GLUT4-positive one (Fig. 2*B*).

In basal and in stimulated cells no GLUT4 was detected in the sarcoplasmic (endoplasmic) reticulum (SR), either in the anastomosing SR network that surrounds the myofibrils (Figs. 2*G* and 3) or in the SR extensions that form junctions with the cell membrane at the sarcolemma (Fig. 1*A*), the ID (Fig. 1*F*), or the T system (Fig. 2 *A–D*). Nor could we detect GLUT4 in the SR of skeletal muscle cells of the rat (not shown). Such labeling described by others (8) probably reflects spurious reactions of the primary antibody, as we discussed before (9).

Table 1 presents quantitative data on the distribution of GLUT4. The total GLUT4 labeling counted per 100 μm^2 of section is about equal to the total nonspecific labeling per 100 μm^2 (35.4 vs. 38.5 particles per 100 μm^2 in basal and 34.6 vs. 35.9 particles per 100 μm^2 in stimulated cells). Therefore, assuming that background distribution is proportional to the random point distribution, we can take the values in the right column of Table 1 as close approximations of the part of GLUT4 labeling values for the various structures in the other two columns that is due to background. This means that in basal cells almost no detectable GLUT4-specific labeling is present at the cell surface. Given the standard errors, the percentage of 0.3 (5.0 minus 4.7) is too low to allow a better estimation than that <1% of GLUT4 resides in the plasma membrane of basal cells. In stimulated cells that value increased to ≈42% (46.6 minus 4.7) of the total. The random point distribution indicates that the background contribution to GLUT4 labeling values for other structures is insignificant. Interestingly, the percentages of specific labeling over the three domains of the plasma membrane in stimulated cells are roughly proportional to the relative areas of these, as assessed by random point analysis. Thus GLUT4 occurs with about the same density in the sarcolemma, ID, and T system. The increase in GLUT4 at the cell surface was quantitatively accounted for by decreases in GLUT4 in T-V elements and the TGR. In this regard, it should be noted that the total GLUT4 labeling in the structures listed in Table 1 was the same in basal and stimulated myocytes (35.4 and 34.6 particles per 100 μm^2, respectively). Knowing that the total amount of GLUT4, as assessed by immunoblotting heart tissue, is unaltered by stimulation (D.E.J., unpublished data), we can conclude that the antibodies used detected intracellular and surface GLUT4 with the same efficiency.

DISCUSSION

In the present study we have immunolocalized GLUT4 in basal and stimulated cardiac muscle from rats. A basal condition of low glucose transport was achieved by fasting (10). Maximal stimulation of glucose transport was achieved by the combination of feeding, exercise, and insulin (1, 2, 10–13). We observed at least 40 times more GLUT4 at the cell surface in stimulated than in basal myocytes (42% vs. <1%). Apparently the surface molecules were recruited from the intracellular pool in T-V elements and TGR, since there the GLUT4 labeling dropped accordingly after stimulation. This magnitude of GLUT4 translocation is of the same order as the increase of glucose transport in cardiac muscle as effected by the additive exercise and insulin stimuli (2, 10, 11, 13). In contrast, using subcellular fractionation, only a 2-fold increase in transporters is observed in heart plasma membranes with stimulation. This difference probably reflects the difficulty in isolating pure plasma membranes from muscle tissue (12–14). Our findings show that translocation of GLUT4 is most likely the major mechanism for stimulation of transport in heart.

GLUT4 labeling was of similar density in plasmalemma, ID, and T system of stimulated cells. Apparently there is no distinction between these surface membranes with respect to translocation of GLUT4. This is of particular interest for the T system. We speculate that, in addition to its role in excitation conductance, this system has a function in regulating the entry of hexoses and possibly other nutrients directly deep into the cells. This may be facilitated by the relatively wide lumen of the T system in cardiac myocytes, the content of which is presumably well mixed during each contraction.

We have also investigated the distribution of GLUT4 in basal and insulin-stimulated brown adipose tissue (6), white adipose tissue, and skeletal (soleus) muscle (J.W.S., unpublished observations) by immunocytochemistry. The results with each of these insulin-responsive tissues are remarkably similar. GLUT4 is virtually excluded from the plasma membrane in the basal state, and a dramatic shift to the plasma membrane from T-V elements and the TGR occurs after stimulation. Based on our observations in brown fat we proposed that in the stimulated state GLUT4 recycles continuously between the cell surface and the endosomal system. In brown fat, endosomal vacuoles are much more prominent than in the myocytes. Consequently, in contrast to the myocytes, the presence of GLUT4 in this organelle during the stimulated state is readily observed (6). However, the enrichment of GLUT4 that we often observed in coated pits suggests that the transporter recycles likewise at each domain of the plasma membrane in stimulated cardiac cells and that stimulation of exocytosis rather than inhibition of endocytosis is the major mechanism for GLUT4 translocation as we postulated for the fat cell (6).

We thank Dr. P. R. Bär (Neurology Department, University of Utrecht) for his advice and for making his animal exercise facilities available for our studies and Dr. A. Sandra (Department of Anatomy, University of Iowa) for critical discussions. This work was supported by grants from the Juvenile Diabetes Foundation (J.W.S.), the Muscular Dystrophy Association (J.W.S. and D.E.J.), and the National Institutes of Health (DK 42503, D.E.J.; DK 25336, G.E.L.).

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